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(54) Title: NICOTINE IMMUNOGENS AND ANTIBODIES AND USES THEREOF

(57) Abstract: Immunogens useful in the production of anti-nicotine antibodies are provided. Anti-nicotine antibodies that immunoreact with the immunogens and nicotine and uses of the immunogens and antibodies in treating nicotine addiction are also provided.

NICOTINE IMMUNOGENS AND ANTIBODIES AND USES THEREOF

Cross-Reference to Related Application

This application is a continuation-in-part of United States Provisional Patent Application Serial No. 60/264,575, filed January 26, 2001, the disclosure of which is incorporated herein by reference.

Technical Field of the Invention

The field of this invention is nicotine addiction. More particularly, this invention pertains to nicotine immunogens, anti-nicotine antibodies and the use of such immunogens and antibodies for treating nicotine addiction.

Background of the Invention

Nicotine is the most widely used addictive drug in the world. As an alkaloid, [(*S*)-(-)-1-methyl-2-(3-pyridyl)pyrrolidine] derived from tobacco leaves, nicotine is available in several forms such as cigarettes, cigars, pipe tobacco, and chewing tobacco. Hence, the drug is intimately linked with cigarette smoking, the leading preventable cause of death in the United States [Nelson, D. E.; Kirkendall, R. S.; Lawton, R. L.; Chrismon, J. H.; Merritt, R. K.; Arday, D. A.; Giovino, G. A. *Morbid. Mortal. Wkly. Rep.* **43**, 1-8 (1994); US Dep. Health Hum. Serv. *Reducing the health consequences of smoking. 25 years of progress. A report of the Surgeon General;* Public Health Services: Rockville, MD, 1989]. Smoking contributes to coronary heart disease, stroke, vascular disease, peptic ulcers, chronic lung diseases and lung cancer, and fetal brain damage and morbidity. Although the dangers of smoking are well known, people continue to smoke.

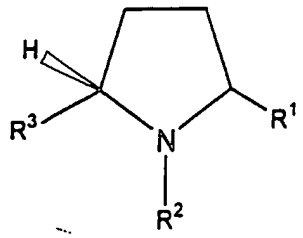
A great deal of evidence supports the view that people continue to smoke because of the addictive effects of nicotine [Benowitz, N. L. *Annu. Rev. Pharmacol. Toxicol.* **36**, 597-613 (1996); Rose, J. E. *Annu. Rev. Med.* **47**, 493-507 (1996)]. However, since nicotine is legally and widely accessible there is relatively little stigma associated with its use, unlike cocaine, heroin and other illicit drugs. Although a large percentage of addicted smokers have

expressed a desire to stop smoking and most who quit do so without treatment, less than 5% of unaided attempts lead to successful long-term abstinence [Hughes, J. R.; Gulliver, S. B.; Fenwick, J. W. et al. *Health Psychol.* **11**, 331-334 (1992); Gritz, E.; Marcus, A.; Carr, C. *J. Psychosoc. Oncol.* **6**, 217-234 (1988)]. Similarly, the two most popular therapies, nicotine gum and transdermal nicotine patches used to slowly wean the user off the drug, have afforded inadequate long-term success rates of <20% [Henningfield, J. E. *New Engl. J. Med.* **333**, 1196-1203 (1995); Hughes, J. R. In *Integrating Behavior Theories with Medication in the Treatment of Drug Dependence* (Eds. Onken, L. S.; Blaine, J. D.; Boren, J. J.); NIDARes Monogr. US GPO: Washington, DC, 1995, pps. 92-109; Palmer, K. J., Buckley, M. M.; Faulds, D. *Drugs* **44**, 498-529 (1992)]. Perhaps because relatively little is known about the specific neuropharmacologic mechanisms underlying nicotine addiction and the response to smoking cessation treatment, no highly effective therapy has been developed.

There is a need to develop a treatment approach to nicotine addiction which does not depend solely on unaided compliance or on the administration of nicotine itself for rehabilitation. One alternative might rely on immunological reagents and the immune system. Recently, we demonstrated the efficacy of immunological strategies with regard to the cocaine abuse problem [Carrera, M. R. A.; Ashley, J. A.; Wirsching, P.; Koob, G. F.; Janda, K. D. *Proc. Natl. Acad. Sci. USA* **98**, 1988-1992 (2001); Carrera, M. R. A.; Ashley, J. A.; Zhou, B.; Wirsching, P.; Koob, G. F, Janda, K. D. *Proc. Natl. Acad. Sci. USA* **97**, 6202-6206 (2000); Carrera, M. Rocio A.; Ashley, J. A.; Parsons, L. H.; Wirsching, P.; Koob, G. F.; Janda, K. D. *Nature* **378**, 727-730 (1995)]. The immune-mediated binding of nicotine would impede its passage into the central nervous system and would result in a suppression of its characteristic actions. In particular, we believe the use of both active immunization (immunoconjugate vaccine) and passive immunization (monoclonal antibodies, mAbs) protocols would provide the most beneficial therapy. Herein, we describe the design and synthesis of haptens necessary to establish an immunopharmacologic arsenal to combat nicotine dependence. Significantly, we have developed a comprehensive program aimed at nicotine as the primary target, as well as the secondary targets nornicotine and cotinine.

Brief Summary of the Invention

In one aspect, the present invention provides a nicotine immunogen. The immunogen has the structure



where R^1 is =O or H, R^2 is H, CH_3 or $(CH_2)_nCONH(CH_2)_2COOH$, and R^3 is H or pyridine and n is an integer from 1 to 10. In preferred embodiments, R^1 is H, R^2 is $(CH_2)_nCONH(CH_2)_2COOH$ and R^3 is pyridine. Preferably, n is from 3 to 7 and, more preferably 5 or 6. In one embodiment, the immunogen is linked to an immunogenic carrier such as keyhole limpet hemocyanin or bovine serum albumin.

In another aspect, this invention provides a method for immunizing an animal against nicotine. In accordance with that method, an animal is administered an effective immunogenic amount of the immunogen of this invention.

In another aspect, this invention provides an antibody that immunoreacts with the immunogen of this invention. Preferably, the antibody also immunoreacts with nicotine. Preferably, the antibody is a monoclonal antibody such as designated herein 3G3, 6C12, 13A3, 1B10, 5E8 or 9D9. An anti-nicotine antibody is made by a process that comprises the step of immunizing an animal with the immunogen of this invention. This invention further provides a method of immunizing an animal against nicotine that includes the step of administering to the animal an effective amount of a subject antibody.

The invention further provides a pharmaceutical composition containing a subject immunogen or antibody together with a physiological diluent.

Brief Description of the Drawings

In the drawings that form a portion of the specification:

FIG. 1 shows the structure of nicotine related tobacco components and their metabolites.

FIG. 2 shows nicotine haptens

FIG. 3 shows a synthetic scheme for making a (*S*)-nicotine hapten: a) ethanol, conc. H_2SO_4, reflux; b) i) NaH, THF, 1-vinyl-2-pyrrolidinone, reflux, ii) aq. HCl, reflux, iii) aq. NaOH; c) $NaBH_4$; d) i) resolution (MTPA salts), ii) H_2, Pd/C, NEt_3, EtOH; e) **14**, DIEA, acetonitrile; f) H_2, Pd/C, MeOH; g) HBTU, N-methylmorpholine, DMF; h) NaI, acetonitrile.

FIG. 4 shows a synthetic scheme for making racemic, and (*S*)- and (*R*)-nornicotine haptens: a) **20**, DIEA, acetonitrile; b) H_2, Pd/C, MeOH; c) benzyl alcohol, p-TsOH, cyclohexane; d) NaI, acetonitrile.

FIG. 5 shows a synthetic scheme for making a (*S*)-cotinine hapten: a) **27**, DIEA, acetonitrile; b) $Hg(OAc)_2$, aq. EDTA, pH 9, dioxane, reflux; c) i) TFA, CH_2Cl_2, ii) **29**, HBTU, N-methylmorpholine, DMF; d) H_2, Pd/C, MeOH; e) di-t-butyldicarbonate, MeOH; f) i) methanesulfonyl chloride, NEt_3, CH_2Cl_2, ii) NaI, acetonitrile; g) benzyl alcohol, DMAP, CH_2Cl_2.

FIG. 6 shows locomotor activity data for rats actively immunized with nicotine immunogen and challenged with nicotine.

FIG. 7 shows locomotor activity data for rats passively immunized with mAb NIC9D9 and challenged with nicotine.

Detailed Description of the Invention

I. The Invention

This invention provides nicotine immunogens, anti-nicotine antibodies and the use of such immunogens and antibodies for immunizing animals against nicotine and treating nicotine addiction.

II. Nicotine Immunogens

Nicotine is a small, haptenic molecule and requires coupling to an immunogenic carrier protein to elicit an immune response. In the past 30 years, a number of reports appeared in the literature that described nicotine haptens and immunoconjugates [Abad, A.; Manclús, J. J.; March, C.; Montoya, A., *Anal. Chem.* **65**, 3227-3231 (1993): Bjercke, R. J.; Cook, G.; Rychlik, N.; Gjika, H. B.; Van Vunakis, H.; Langone, J. J. *J. Immunol. Methods* **90**, 203-213 (1986); Castro, A.; McKennis Jr., H.; Monji, N.; Bowman, E. R. *Biochem. Arch.* **1**, 205-214 (1985); Castro, A.; Monji, N.; Hacer, A.; Bowman, E. R.; McKennis Jr., H. *Biochem. Arch.* **1**, 173-183 (1985); Langone, J. J.; Van Vunakis, H. *Methods Enzymol.* **84**, 628-640 (1982); Matsushita, H.; Noguchi, M.; Tamaki, E. *Biochem. Biophys. Res. Commun.* **57**, 1006-1010 (1974); and Langone, J. J.; Gjika, H. B.; Van Vunakis, H., *Biochem.* **12**, 5025-5030 (1973)].

That work was aimed primarily at the development of enzyme-linked immunosorbent assays (ELISA) for more convenient detection of nicotine in a variety of media such as blood, urine, and smoke residue. The majority of these studies utilized polyclonal immunoglobulins (antisera) from rabbits/goats. In a few examples, murine monoclonal antibody (mAb) preparations were examined. While the data in most cases were acceptable with regard to antibody affinity and specificity, erratic and variable results were noted. In light of some of these observations, there is clearly room for improvement with regard to both hapten design and the quality of anti-nicotine immune responses and antibody preparations. Notably, even slight improvements would afford enhanced performance in immunopharmacotherapy protocols.

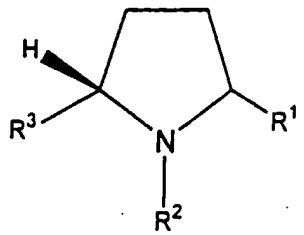
For optimum results, the design and synthesis of a nicotine hapten requires attention to stereochemistry, ionic/acid-base properties, and the site of attachment and characteristics of a linker moiety. Nicotine, which occurs as the (S)-configuration in nature (e.g. tobacco), contains two rings with an asymmetric center. There are two sites of basicity: the pyridyl and pyrrolidyl nitrogens. Nicotine is expected to carry a positive charge at physiological pH. Hence, an optimal immunogen (i.e., hapten) should incorporate a linker when coupled as the immunoconjugate in such a way as to present both ring nitrogens and have the proper stereochemistry and charge characteristics. Exemplary and preferred nicotinic compounds useful in the design of immunogens are shown in FIG. 1. Those compounds are (S)-(-)-nicotine, **Compound 1,** the structurally related nornicotine, **Compound 2**, present in tobacco and a metabolite, cotinine, **Compound 3**, the major primary metabolite of nicotine, and N-methylpyrrolidine, **Compound 4,** a minor tobacco component.

The general approach to designing nicotine immunogens is to maintain unchanged the significant structural and stereochemical features of nicotine as it occurs in tobacco. If possible, no additional functionality is imparted to the immunogen that is not in nicotine. Any changes that are made should be weakly immunogenic and a linker of appropriate length is attached in a position to present all important recognition elements. With regard to the nicotine problem, implementation of the above strategy first requires an immunogen with the natural (S)-configuration. Although an immune response would be expected against both antipodes of a racemic immunogen, stereospecificity is likely enhanced using the single (S)-antipode. Only an immune response against (S)-(-)-nicotine. (R)-(+)-nicotine does not have significant pharmacological activity. Therefore, it is not necessary to bind any trace amounts of this isomer during therapy. Consequently, avoidance of even a few percent of cross-reactivity with this isomer would benefit binding of the desired target (S)-(-)-nicotine.

Second, the immunogen should contain an alkyl linker at the position of the methyl group on the pyrrolidine nitrogen. In this way, all the characteristics of nicotine remain intact for display to the immune system: stereochemistry, two unaltered rings, charge, and the important pyrrolidyl methyl group mimicked as part of an alkyl linker. The length and

chemical composition of the linker are selected in order to afford recognition of the nicotine framework in its entirety.

Thus, an immunogen of this invention corresponds to formula I, below.



In Formula I, R^1 is =O or H, R^2 is a linker moiety and R^3 is H or a 5- or 6-membered aromatic. A preferred linker moiety is $(CH_2)_nCONH(CH_2)_2COOH$ or $CO(CH_2)_nNH(CH_2)_2COOH$ where n is an integer from 1 to 10. Exemplary and preferred aromatics are phenyl, pyridine, and other six membered aromatics.

In contrast to the present immunogens, haptens employed by others were derived from 2-aminonicotine (**Compound A**), 6-aminonicotine (**Compound B**), and trans-3'-hydroxymethylnicotine (**Compound C**), all as shown in FIG. 2. Further, all prior haptens were racemic compounds. When nicotine antibodies were prepared using **Compound A** or **B** there was a strong cross-reaction with N-methylpyrrolidine, **Compound 4**, a constituent of tobacco smoke [Langone, J. J.; Gjika, H. B.; Van Vunakis, H., *Biochem.* **12**, 5025-5030 (1973)]. Using similar haptens, others observed only limited binding of **Compound 4** [Castro, A.; McKennis Jr., H.; Monji, N.; Bowman, E. R., *Biochem. Arch.* **1**, 205-214 (1985) and Castro, A.; Monji, N.; Hacer, A.; Bowman, E. R.; McKennis Jr., H., *Biochem. Arch.* **1**, 173-183 (1985)].

Matsushita et al. believed that increased exposure of the pyrrolidine ring of a nicotine hapten would result via coupling at the pyridine nucleus [Matsushita, H.; Noguchi, M.; Tamaki, E. *Biochem. Biophys. Res. Commun.* **57**, 1006-1010 (1974)]. These workers considered **Compound B** as the most satisfactory structure and that the antibodies obtained were more specific than those elicited using **Compound C** preferred by Langone et al. However, racemic **Compound B** conjugated using a diazotized p-aminobenzoyl linker afforded antisera

highly specific for natural (S)-(-)-nicotine with only 5% cross-reaction of the (R)-(+)-isomer [Matsushita, H.; Noguchi, M.; Tamaki, E. *Biochem. Biophys. Res. Commun.* **57**, 1006-1010 (1974)]. While these data might be correct and even rather favorable, such a result is difficult to readily explain. Langone et al. prepared nicotine antisera and mAbs derived from **Compound C**, perhaps the most widely recognized, studied and accepted hapten for anti-nicotine antibody production [Bjercke, R. J.; Cook, G.; Rychlik, N.; Gjika, H. B.; Van Vunakis, H.; Langone, J. J., *J. Immunol. Methods* **90**, 203-213 (1986); Langone, J. J.; Van Vunakis, H., *Methods Enzymol.* **84**, 628-640 (1982); and Matsushita, H.; Noguchi, M.; Tamaki, E. *Biochem. Biophys. Res. Commun.* **57**, 1006-1010 (1974)]. Affinities on the order of 10^{-8} M were reported for various mAbs. Yet, other workers using the same hapten isolated mAbs with only micromolar K_d values for nicotine and increased cross-reactivities versus some metabolites and tobacco components [Abad, A.; Manclús, J. J.; March, C.; Montoya, A., *Anal. Chem.* **65**, 3227-3231 (1993)]. Castro and coworkers examined various linkers lengths and compositions using **Compounds A** and **B** to assess effects on affinity and specificity, and somewhat surprisingly observed no significant differences [Castro, A.; McKennis Jr., H.; Monji, N.; Bowman, E. R., *Biochem. Arch.* **1**, 205-210 (1985)]. In addition, some unusual time-dependent binding behavior of compounds in cross-reactivity studies were also described.

A preferred immunogen is **Compound 10** (**NIC**), which was synthesized with high enantiomeric excess. To accomplish this, a literature procedure was used to prepare nornicotine with the (S)-(-) stereochemistry corresponding to natural nicotine in ≥98% optical purity [Jacob, P. III., *J. Org. Chem.* **47**, 4165-4167 (1982)]. Coupling of **Compound 10** to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) afforded the conjugates. Consequently, hapten **10** (code: **NIC**) was synthesized with the (*S*)-configuration corresponding to natural nicotine (FIG. 3). To this end, the formation of 5-bromomyosmine **Compound 7** occurred via the base-mediated condensation of ethyl 5-bromonicotinate **Compound 6** with *N*-vinyl-pyrrolidinone, followed by *in situ* acid-catalyzed hydrolysis of the β-keto-*N*-vinyllactam, decarboxylation, and cyclization. The isolated imine was then reduced with sodium borohydride to afford 5-bromonornicotine **Compound 8**.

Nornicotine **Compound 2b** was then obtained with the (*S*)-(-) stereochemistry in ≥98% ee via a classical resolution of the racemic **Compound 8**.
In the process, the (*R*)-isomer **Compound 2c** was also procured with a similar purity. The reaction with linker **Compound 14** proceeded with a good yield of monoalkylation. We anticipated that, after purification of the intermediate benzyl ester, hydrogenation would afford isolated **NIC** ready for use. However, somewhat surprisingly, ~25% of the ring-opened, 2'-C-N cleavage product **Compound 15** was also produced, even at one atmosphere of hydrogen and short reaction times, that necessitated purification of the final hapten. Benzylic amines are generally more resistant to hydrogenolysis than benzyl esters and acidic media are often necessary to facilitate the reaction. In this case, the benzylic-like C-N bond adjacent to the pyridyl ring was sufficiently labile to compete with hydrogenolysis of the benzyl ester C-O bond. Although there was no by-product in the conversion of **Compound 8** to nornicotine, the reaction conditions were different and incorporated triethylamine. Since we omitted a base in the reaction of **Compound 9** in order to avoid salt formation of the **NIC** hapten, perhaps the more acidic environment, especially after benzyl ester hydrogenolysis, activated the C-N bond towards cleavage.

All previously reported nicotine haptens contained short linkers, at either the pyridyl or pyrrolidyl ring carbons, and were racemic in nature. The linker used here, ~12 Å in length and with an internal amide bond we recently found useful for hapten immune responses, should yield complete and high affinity recognition of all features of the nicotine molecule.

Nornicotine **2** occurs in tobacco as a mixture of (*R*)- and (*S*)-enantiomeric forms. Although present as only 2% of total alkaloids, the compound is pharmacologically active in the central nervous system similar to nicotine [Jacob, P., III; Yu, L.; Liang, G.; Shulgin, A. T.; Benowitz, N. L. *J. Chromatogr.* **619**, 49-611 (1993); Saitoh, F.; Noma, M.; Kawashima, N. *Phytochemistry* **24**, 477-480 (1985); Bardo, M. T.; Bevins, R. A.; Klebaur, J. E.; Crooks, P. A.; Dwoskin, P. L. *Pharmacol. Biochem. Behav.* **58**, 1083-1087 (1997); Crooks, P. A.; Dwoskin, L. P. *Biochem. Pharmacol.* **54**, 743-753 (1996); Dwoskin, L. P.; Teng, L.; Buxton, S. T.; Ravard, A.; Deo, N.; Crooks, P. A. *Eur. J. Pharmacol.* **276**, 195-199 (1995); Dwoskin, L. P.; Buxton, S. T.; Jewell, A. L.; Crooks, P. A. *J. Neurochem.* **60**, 2167-2174 (1993);

Copeland J. R.; Adem, A.; Jacob, P., III; Nordberg, A. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **343**, 123-127 (1991); Risner, M. E.; Cone, E. J.; Benowitz, N. L.; Jacob, P., III. *J. Pharmacol. Exp. Ther.* **244**, 807-813 (1988)]. Both optical isomers of nornicotine have similar binding affinities to the nicotinic acetylcholine receptor, but there is evidence that the enantiomers differ in their behavioral and pharmacological activities [Bardo, M. T.; Bevins, R. A.; Klebaur, J. E.; Crooks, P. A.; Dwoskin, P. L. *Pharmacol. Biochem. Behav.* **58**, 1083-1087 (1997); Crooks, P. A.; Dwoskin, L. P. *Biochem. Pharmacol.* **54**, 743-753 (1996); Dwoskin, L. P.; Teng, L.; Buxton, S. T.; Ravard, A.; Deo, N.; Crooks, P. A. *Eur. J. Pharmacol.* **276**, 195-199 (1995); Dwoskin, L. P.; Buxton, S. T.; Jewell, A. L.; Crooks, P. A. *J. Neurochem.* **60**, 2167-2174 (1993); Copeland J. R.; Adem, A.; Jacob, P., III; Nordberg, A. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **343**, 123-127 (1991); Risner, M. E.; Cone, E. J.; Benowitz, N. L.; Jacob, P., III. *J. Pharmacol. Exp. Ther.* **244**, 807-813 (1988)]. Nornicotine also accounts for ~8% of metabolized nicotine in both humans and rats and has a plasma half-life of about eight hours which is longer than the average two-hour half-life of nicotine [Curvall, M.; Kazeni, V. E. In *Nicotine and Related Alkaloids: Absorption, Distribution, Metabolism, and Excretion.* (Eds. Gorrod, J. W.; Wahren, J.); Chapman Hall: London, 1993, pp. 147-179; Kyerematen, G. A.; Morgan, M.; Chattopadhyay, B.; DeBethizy, J. D.; Vesell, E. S. *Clin. Pharmacol. Ther.* **48**, 641-651 (1990); Cundy, K. C.; Crooks, P. A. *J. Chromatogr. B Biomed. Sci. Appl.* **306**, 291-230 (1984); McKennis, H.; Turnbull, L. B.; Schwartz, S. L. *J. Biol. Chem.* **237**, 541-545 (1962)].

Most significant, animal behavioral studies of rats demonstrate that nornicotine effects locomotor activity and is self-administered [Bardo, M. T.; Green, T. A.; Crooks, P. A.; Dwoskin, L. P. *Psychopharmacology* **146**, 290-296 (1999); Dwoskin, L. P.; Crooks, P. A.; Teng, L.; Green, T. A.; Bardo, M. T. *Psychopharmacology* **145**, 442-451 (1999)].

At first, the hapten **Compound 16a** (code: **NOC**) was synthesized starting from *rac*-nornicotine **Compound 2a** (Fig. 3). However, preliminary tests indicated the absence of a viable immune response against the hapten in accord with previous hypotheses. Therefore, separate (*S*)-**NOC** and (*R*)-**NOC** haptens were prepared in order to obtain antibodies specific for each isomer of nornicotine. Hence, linker **Compound 20** was introduced onto the nornicotine framework using reaction chemistry similar to that employed for the preparation

of **Compound 9**. Hydrogenation of **Compound 16a-c** afforded **NOC** haptens which required purification, again due to the formation of a ring-opened product analogous to **Compound 15**. Although the **NOC** hapten is structurally analogous to **NIC**, the "tuning" of the linker length should be critical. We anticipate that the shorter and less flexible linker will afford a substantial fraction of antibodies that recognize only the pyridyl and pyrrolidyl rings and not the alkyl-chain region. Consequently, binding should be more specific for nornicotine rather than the nicotine structure bearing a methylated nitrogen.

Cotinine **Compound 3** accounts for 70-80% of metabolized nicotine and has a long half-life of 16-20 hours [Jacob, P., III; Benowitz, N. L. *Clin. Pharmacol. Ther.* **56**, 483-493 (1994); Jacob, P., III; Benowitz, N. L.; Shulgin, A. T. *Pharmacol. Biochem. Behav.* **30**, 249-253 (1988); Benowitz, N. L.; Kuyt, F.; Jacob, P., III; Jones, R. T.; Osman, A. L. *Clin. Pharmacol. Ther.* **34**, 604-611 (1983); Benowitz, N. L.; Hall, S. M.; Herning, R. I. *N. Engl. J. Med.* **309**, 139-142 (1983)]. In addition, cotinine levels can average 15-fold higher than nicotine during smoking or nicotine replacement therapy [Jacob, P., III; Benowitz, N. L. *Clin. Pharmacol. Ther.* **56**, 483-493 (1994)]. Apparently, cotinine also 1) has an effect on nicotinic cholinergic receptors *in vitro*, 2) influences release of neurotransmitters, 3) inhibits androgen biosynthesis, and 4) possibly contributes to the lower blood pressure of smokers during nonsmoking intervals [Dwoskin, L. P.; Teng, L.; Buxton, S. T.; Crooks, P. A. *J. Pharmacol. Exp. Ther.* **288**, 905-911 (1999); Benowitz, N. L.; Sharp, D. S. *Circulation* **80**, 1309-1312 (1989); Yeh, J.; Barbieri, R. L.; Friedman, A. J. *J. Steroid Biochem.* **33**, 627-630 (1989); Fuxe, K.; Everitt, B. J.; Hokfelt, T. *Pharmacol. Biochem. Behav.* **10**, 671-677 (1979)]. Notably, cotinine is reported to effect the pharmacology of nicotine withdrawal and could promote cardiovascular and endocrine effects and withdrawal symptoms after nicotine abstinence [Keenan, R. M.; Hatsukami, D. K.; Pentel, P. R.; Thompson, T. N.; Grillo, M. A. *Clin. Pharmacol. Ther.* **55**, 581-590 (1994)].

Hence, inclusion of cotinine as a target is warranted in an immunotherapeutic approach to the nicotine problem.

For cotinine-specific antibodies, the (*S*)-hapten **Compound 24** (code: **COT**) was synthesized starting from **Compound 2b** (Fig. 4). The central transformation was the

Wenkert oxidation of the amine **Compound 21** using conditions similar to those in the literature for the conversion of nicotine to cotinine [Wenkert, E.; Angell, E. C. *Synth. Commun.* **18**, 1331-1337 (1988)]. The stereochemical integrity should not be effected, since no racemization occurs in this reaction.

Modification of the linker chemistry by using **Compound 27** was necessary to provide a compound that was stable during the basic conditions of the oxidation. The benzyl ester-containing linker in **Compound 9** decomposed and gave products that could not be purified. Interestingly, unlike in the **NIC** synthesis, the hydrogenation to produce **COT** was nearly quantitative. There was no cleavage of the 5'-C-N bond likely due to deactivation by the pyrrolidone ring and the absence of a basic nitrogen as in **Compound 9**. Notably, the final linker in **Compound 24** differs from that in **Compound 10** by only one bond length while maintaining the desirable internal amide bond.

An immunogen is typically operatively linked to an immunogenic carrier before immunization of an animal. Useful carriers are well known in the art and are generally proteins themselves. Exemplary of such carriers are keyhole limpet hemocyanin (KLH), edestin, thyroglobulin, albumins such as bovine serum albumin or human serum albumin (BSA or HSA, respectively), red blood cells such as sheep erythrocytes (SRBC), tetanus toxoid, cholera toxoid as well as polyamino acids such as poly(D-lysine:D-glutamic acid), and the like. The choice of carrier is more dependent upon the ultimate intended use of the antigen than upon the determinant portion of the antigen, and is based upon criteria not particularly involved in the present invention. For example, if the conjugate is to be used in laboratory animals, a carrier that does not generate an untoward reaction in the particular animal should be selected. The carrier-hapten conjugate is dissolved or dispersed in an aqueous composition of a physiologically tolerable diluent such as normal saline, PBS, or sterile water to form an inoculum. An adjuvant such as complete or incomplete Freund's adjuvant or alum can also be included in the inoculum. The inoculum is introduced as by injection into the animal used to raise the antibodies in an amount sufficient to induce antibodies, as is well known.

III. Anti-nicotine Antibodies

The present invention provides antibodies that immunoreact with an immunogen of this invention. An antibody can be a polyclonal or monoclonal antibody or an immunoreactive fragment thereof. Means for making polyclonal and monoclonal antibodies are well known in the art. Still further an antibody of this invention can be a recombinant antibody. Means for making recombinant antibodies are well known in the art. By way of example, immunoglobulin mRNA can be cloned from specific hybridomas and expessed using phage display. A recombinant antibody can be manipulated or mutated so as to improve its binding ability to an antigen such as nicotine. Means for such manipulation/mutation are also well known in the art. An antibody of this invention also cross reacts with nicotine. Preferably, the antibody cross reacts with S-(-), but not R-(+) nicotine.

Compound 10-KLH (**NIC**-KLH) was used for immunization and **Compound 10**-BSA (**NIC**-BSA) for ELISA. The **NIC**-KLH was also used in rat behavioral models to study the efficacy of vaccination protocols against nicotine addiction. Also, **NIC**-KLH was used to immunize mice in a highly consistent fashion and thereby produce both a quality, reproducible immune response as well as mAbs with excellent affinity and specificity for nicotine. All of the mAbs were fully characterized with respect to purity, isotype, and hybridoma production yields. We have used competition ELISA to select mAbs and obtain preliminary binding constants and equilibrium dialysis (radiolabeled **Compound 1** was synthesized in our laboratory as the tritiated N-methyl derivative from (S)-(-)-nornicotine and [^{3}H]-methyl iodide) to obtain more refined binding constants ($K_d \sim 10^{-7}$ M) (Table 1). Both ELISA and competition ELISA were used to assess specificity. Preliminary studies indicated no cross-reactivity with (R)-(+)-nicotine, nornicotine (R or S), cotinine, or N-methylpyrrolidine at 100-1000-fold molar excesses of these compounds.

Table 1. Data for some anti-nicotine mAbs derived from NIC-KLH.

NIC mAb	isotype (IgG)	K_d ($M \times 10^7$)	specificity[a] (%)
3G3	κγ1	3.0	0.30, nd
6C12	κγ1	2.7	0.15, nd
13A3	κγ2a	1.6	0.10, nd
1B10	κγ1	2.5	0.20, nd
5E8	κγ1	3.4	0.65, nd
9D9	κγ1	2.0	0.055, nd

[a]Determined by enzyme-linked immunosorbent assay (ELISA) using NIC-BSA; percent cross-reactivity (**2b**, **3**) relative to the amount of free nicotine that produces 50% decrease in mAb binding; nd = not detectable (no change in ELISA reading at 1000-fold molar excess of **3** compared to nicotine).

The K_d values for nicotine binding surpassed all previously reported examples except those of Langone et al. that were ~10-fold better [Bjercke, R. J.; Cook, G.; Rychlik, N.; Gjika, H. B.; Van Vunakis, H.; Langone, J. J. *J. Immunol. Methods* **90**, 203-213 (1986)]. However, the same hapten employed by these workers was used by others and afforded mAbs that bound nicotine ~100-fold less tightly [Abad, A.; Manclús, J. J.; March, C.; Montoya, A. *Anal. Chem.* **65**, 3227-3231 (1993)]. Hence, the characteristics of this hapten, or its immunoconjugates, might not be conducive for achieving consistent titers which would be a drawback for therapeutic purposes. Although our anti-nicotine mAbs had lower affinities, the specificity for nicotine relative to nornicotine and cotinine was improved by several-fold compared to the Langone mAbs which lends support to aspects of our hapten design. Consequently, **2**, **3**, and **4** should not compete with nicotine for antibody binding sites and therefore would not pose a problem during treatment.

The murine mAbs can be "humanized" via several techniques well known in the art [James, K. Human monoclonal antibodies. In: *Handbook of Experimental Pharmacology : The Pharmacology of Monoclonal Antibodies*, Rosenberg, M.; Moore, G. P., eds. New York: Springer-Verlag, vol. 113, pp. 3-19, 1994; Padlan, E. A., *Mol. Immunol.* **28**, 489-498 (1991); Daugherty, B. L.; DeMartino, J. A.; Law, M.-F.; Kawka, D. W.; Singer, I. I.; Mark, G. E., *Nucleic Acids Res.* **19**, 2471-2476 (1991); and Riechmann, L.; Clark, M.; Waldmann, H.; Winter, G., *Nature* **332**, 323-327 (1988)]. We recently completed the "humanization" of an anti-cocaine antibody in which the binding properties were similar to the starting murine mAb. In addition, our methodology for selecting antibodies of desired specificity from combinatorial libraries would make human mAbs (scFvs or Fabs) directly available. If desired, protein engineering would then afford human IgG constructs. Access to humanized or fully human mAbs makes clinical application feasible.

IV. Methods of Immunization

A. Active Immunization

One goal in an immunological approach aimed at the abatement of nicotine addiction is the *de novo* design of a vaccine. In principle, vaccination would impart to the host's immune system the ability to defend against the acute psychostimulant and toxic effects of nicotine and its powerful reinforcing properties. Importantly, an active immunization against nicotine offers a means of blocking the actions of the drug by preventing it from entering the central nervous system and should have fewer side effects than treatments based on manipulation of central neurotransmitter function. Thus, immunopharmacotherapy may offer a nontoxic, substance-specific strategy that should not affect normal neurochemical physiology, presenting a solid scientific approach for the treatment of nicotine dependence.

The effect of immunization with NIC-KLH on acute nicotine-induced locomotor activity was investigated. Male Wistar rats were tested in photocell cages after s.c. administration of nicotine (0.50 mg/kg; 1 ml/kg) to determine pre-immunization drug response (baseline). Experimental animals were immunized i. p. three days later with NIC-KLH in an emulsion with an adjuvant (Ribi). Control animals were injected with a similar emulsion containing KLH solution only. This treatment was followed by boosts at 21 and 35

days. Rats were then challenged with systemic nicotine and their locomotor responses were again measured (FIG. 6).

The initial pre-immunization nicotine injections resulted in the classic inhibitory effect on locomotor activity induced by acute treatment of the drug (Fig. 6A). With repeated administrations of nicotine, tolerance to the initial depressant effect developed, and by the sixth nicotine injection, the activating effects of the drug were observed (Fig. 6B). The first nicotine challenge resulted in a non-significant difference in locomotor activity (Fig. 6C) [$F(1,14) = 3.125$; $P < 0.099$]. This result may be interpreted as a re-emergence of the initial depressant effect of nicotine in control but not experimental rats. The last nicotine challenge produced a significant difference between groups in locomotor activity [$F(1,14) = 6.392$; $P < 0.024$]. Compared to baseline values, experimental rats as a group showed a 45% decrease in the ambulatory measure (crossovers) (NIC-KLH: 542.63 ± 59.85; 307.38 ± 49.13), whereas controls showed a decrease of 16% (KLH: 516.63 ± 64.81; 437.50 ± 61.48) (Fig. 6D). These results, although not dramatic, indicate that inoculation with NIC-KLH significantly suppressed the psychosmotor effects of nicotine as compared to controls. We intend to use a new vaccine formulation (*vide supra*) that should afford better results.

B. Passive Immunization

We are in the process of accumulating large amounts of our best anti-nicotine mAbs elicited with NIC-KLH for *in vivo* experimentation in rats. Briefly, we are currently using Integra Biosciences CELLine flasks for *in vitro* production of mAb NIC9D9 (Table 1). Each CL-1000 flask can produce 100-200 mg of mAb per month. To produce the ~3 grams of antibody we estimate will be necessary to run all behavioral paradigms, we will set up 6 CL-1000's and maintain cell cultures for ~3 months. We inoculate the cell chamber with 1-1.5 x 10^8 hybridoma cells (generally 200 ml of between 5-7 x 10^5 cells/ml) in 15 ml of complete media (RPMI with 2 mM L-glutamine, 10 mM Hepes, 1 mM Na pyruvate, 100 μg/ml gentamycin sulfate, 500 units penicillin, 500 units streptomycin supplemented with 20% fetal calf serum). The basal chamber has one liter of complete media without serum. After one week, the cell density and viability is checked. We harvest and feed the cell compartment, keeping the viability over 50% and the density 5 x 10^6 cells/ml, and change the basal media.

The harvested cells (generally 15-20 ml) are centrifuged at 1500 rpm for 10 min. The supernatant is filtered through a 0.22 μm filter and purified through a protein G column. The concentration of antibody in the cell supernatant is usually ~1-2 mg/ml. As the culture gets more established in the second and third month, we can harvest two times per week with 15-20 ml/harvest and obtain 400 mg per CL-1000.

The administration of antibodies to suppress the effects of nicotine would roughly parallel our work on cocaine immunopharmacotherapy [Carrera, M. R. A.; Ashley, J. A.; Wirsching, P.; Koob, G. F.; Janda, K. D., *Proc. Natl. Acad. Sci. USA* **98**, 1988-1992 (2001); Carrera, M. R. A.; Ashley, J. A.; Zhou, B.; Wirsching, P.; Koob, G. F, Janda, K. D., *Proc. Natl. Acad. Sci. USA* **97**, 6202-6206 (2000); and Carrera, M. Rocio A.; Ashley, J. A.; Parsons, L. H.; Wirsching, P.; Koob, G. F.; Janda, K. D., *Nature* **378**, 727-730 (1995)]. The passive administration of anti-nicotine antibodies should prove beneficial to reduce serum levels and attenuate "toxic" (cardiovascular, metabolic, endocrine) effects, or as (bi)weekly pharmacotherapy during smoking cessation programs. The latter could entail self-injection of mAb to maintain a high circulating level of antibody. Significantly, in a more novel and user-palatable approach, it should be possible to establish passive mucosal protection against nicotine in the respiratory tract through the use of aerosolized immunoglobulin [Crowe, J. E., Jr.; Murphy, B. R.; Chanock, R. M.; Williamson, R. A.; Barbas, C. F., III; Burton, D. R., *Proc. Natl. Acad. Sci. USA* **91**, 1386-1390 (1994)]. This method would be particularly applicable to the nicotine dependence problem since the vast majority of users obtain nicotine by smoking.

In a preliminary experiment, we acquired locomotor activity data for rats passively immunized with mAb NIC9D9 (Fig. 7). Male Wistar rats were prepared with intrajugular catheters and allowed a 7-day recovery period. All animals were treated with a subcutaneous (s.c.) nicotine (0.28 mg/kg; 1 ml/kg body weight) for five consecutive days and observed in photocell cages after administration to determine pre-immunization drug response (baseline). On testing days, experimental animals were infused i.v. with 5, 25 or 50 mg/kg of NIC9D9 in a volume of approximately 1.5 ml/kg. Control rats were treated with an equivalent volume of

physiological saline (i.v.). Approximately 30 min after infusions, all animals received s.c. nicotine injection (0.28 mg/kg) and their locomotor responses were assessed for the next 90 min. All locomotor activity test sessions were preceded by a 90 min habituation session following s.c. saline injection (1 ml/kg). The dose-response to NIC9D9 was produced by testing each dose two weeks apart, in order to avoid carry-over effects. All locomotor data reported refer to the ambulatory measure or crossovers.

The initial pre-immunization nicotine injections resulted in the classic inhibitory effect on locomotor activity induced by acute treatment of the drug. With repeated administrations of nicotine, tolerance to the initial depressant effect developed, and by the sixth nicotine injection, the activating effects of the drug were observed (Figure 7A). This last pre-immunization nicotine challenge resulted in a nonsignificant difference in locomotor activity [$F(1,14) = 0.18$; $P < 0.895$]. Passive transfer with NIC9D9 (5 mg/kg) in the experimental group resulted in a significant decrease in locomotor activity compared to controls (Figure 7B) during the first 20 min of the session [$F(1,14) = 6.757$; $P < 0.020$]. This effect was optimized by increasing doses of the mAb. After i.v. infusion of NIC9D9 (25 mg/kg), differences in locomotion between groups were observed throughout the first 40 min of the session [$F(1,14) = 18.214$; $P < 0.0008$] (Figure 7C). The maximum effect was obtained upon treatment with the highest mAb dose (50 mg/kg), where differences in the ambulatory measure persisted throughout the first 40 min of the session [$F(1.14) = 37.376$; $P < 0.000$] (Figure 7D) In addition, a dramatic decrease during the initial 20 min of testing of the ambulatory locomotor responses were observed. Compared to baseline values, experimental rats as a group showed a 66.9% decrease in locomotor activity (NIC9D9: 703.38 ± 111.88; 308.75 ± 57.51), whereas controls showed a decrease of 3.4% (SAL: 688.13 ± 111.10; 740 ± 117.62).

These results indicate that passive transfer with the mAb NIC9D9 dose-dependently suppressed the psychosmotor effects of nicotine as compared to controls. Furthermore, the data provide a solid foundation for continuing studies of passive immunization as a means to address nicotine dependence. We believe this approach will be highly amenable to the treatment of nicotine dependence in the human condition.

Eventually, in humans, we can also consider a variation on the passive protection scenario. From the standpoint of scientific rationale and user compliance, we believe the inhalation of anti-nicotine antibodies via compact, portable inhalers might constitute the single most effective means to treat the problem of nicotine addiction. As we describe in this proposal, binding nicotine in the systemic circulation using a vaccine or with passive antibodies should be an effective therapy. However, a direct blockade in the pulmonary system, together with systemic protection, or even as a stand-alone treatment, would greatly suppress the concentrations of nicotine that rapidly reach the brain during the action of smoking. Hence, such a passive immunization approach might be particularly effective by keeping nicotine below a threshold concentration required for reinforcement.

While both a nicotine vaccine and an anti-nicotine mAb are part of our therapeutic strategy, we believe passive immunization protocols would be most practical with regard to **2** and **3**. Certainly, the binding and blockade of nicotine is of paramount importance for therapy. However, the additional treatment with a "cocktail" of mAbs specific for nornicotine and cotinine could have important ramifications with regard to reducing reinforcement and relapse potential, especially for some individuals. The haptens described provide the essential elements in a program of immunopharmacotherapy that offers a new avenue in the challenging battle against nicotine addiction.

IV. Pharmaceutical Composition

The present invention further provides a pharmaceutical composition. The pharmaceutical composition includes a compound of this invention (immunogen, antibody) together with a physiologically tolerable carrier.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified.

The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient.

The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-aminoethanol, histidine, procaine and the like. Particularly preferred are the salts of TFA and HCl.

Physiologically tolerable carriers are well known in the art. Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes. Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such

additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions.

The Examples that follow illustrate preferred embodiments of the present invention and are not limiting of the specification and claims in any way.

EXAMPLE 1: **General Methods.** ^{1}H and ^{13}C NMR spectra were measured at 400 MHz on a Brucker AMX-400 spectrometer. Chemical sifts (ppm) were reported relative to internal $CDCl_3$ (^{1}H, 7.26 ppm and ^{13}C, 77.0 ppm) and CD_3OD (^{1}H, 3.30 ppm and ^{13}C, 49.2 ppm). HRMS spectra were recorded using electrospray ionization (ESI) or MALDI techniques. Glassware and solvents were dried by standard methods. Flash chromatography was performed on silica gel 60 (230-400 mesh) and thin-layer chromatography on glass plates coated with a 0.02 mm layer of silica gel 60 F-254. The *rac*-nornicotine **2a** and other chemical reagents and solvents were from Aldrich Chem. Co., unless otherwise noted, and used without further purification.

EXAMPLE 2: **Ethyl 5-bromonicotinate Compound 6.** A solution of 5-bromonicotinic acid **Compound 5** (20.6 g, 102 mmol) in a mixture of ethanol (400 mL) and conc. sulfuric acid (5 mL) was stirred at reflux under nitrogen for 18 hr. The ethanol was removed evaporated and the resulting white residue dissolved in water. The aqueous solution was made basic to pH 8 with sat. sodium bicarbonate and extracted with ether. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to give **Compound 6** as a pale yellow oil (22 g, 94% yield). ^{1}H NMR ($CDCl_3$) δ: 9.08 (d, J = 1.8 Hz, 1H), 8.79 (d, J = 2.4 Hz, 1H), 8.38 (dd, J = 1.8, 2.4 Hz, 1H), 4.38 (q, J = 7.0 Hz, 2H), 1.38 (t, J = 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 163.9, 154.3, 148.7, 139.3, 127.5, 120.5, 61.8, 14.1. HRMS (MALDI-FTMS): calcd for $C_8H_9BrNO_2$ (MH^+) 229.9817, found 229.9816.

EXAMPLE 3: **3-Bromo-5-(4, 5-dihydro-3*H*-pyrrol-2-yl)-pyridine (5-bromomyosmine) Compound 7.** Sodium hydride (2.4 g, 60 mmol, 60% dispersion in oil) in a 3-neck flask was washed with three 20 mL portions of hexane. The flask was fitted with a reflux condenser, flushed with nitrogen, and charged with THF (85 mL). A solution of **Compound 6** (10.6 g, 46.1 mmol) and 1-vinyl-2-pyrrolidinone (5.5 g, 49.5 mmol) in THF (15

mL) was added in one portion. The mixture was stirred and refluxed for 1 hr, and then cooled to r.t. A solution of conc. HCl (8 mL) in water (12 mL) was added and the THF removed on a rotary evaporator. Additional conc. HCl (12 mL) and water (24 mL) were added and the mixture heated at reflux overnight. In an ice-cooled bath, the solution was made basic with conc. aqueous NaOH, which resulted in precipitation of the crude product, and then extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with water and brine and evaporated. The residue was purified by chromatography on silica gel eluting with CH_2Cl_2/acetone (19:1) to **Compound 7** as a pale yellow solid (7.4 g, 72% yield). ^{1}H NMR ($CDCl_3$) δ: 8.82 (d, J = 1.8 Hz, 1H), 8.65 (d, J = 2.4 Hz, 1H), 8.29 (t, J = 2.1 Hz, 1H), 4.06-4.01 (m, 2H), 2.91-2.85 (m, 2H), 2.07-1.98 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 169.7, 152.0, 146.9, 137.0, 131.5, 120.8, 61.6, 34.7, 22.4. MS (ESI): $C_9H_9BrN_2$ (Fw 224/226), *m/z* 225/227 (MH^+).

EXAMPLE 4: 3-Bromo-5-(2-pyrrolidinyl)-pyridine (5-bromonornicotine) Compound 8. Sodium borohydride (4.5 g, 119 mmol) was added portionwise over 10 min with vigorous stirring to a solution of **Compound 7** (12.1 g, 53.8 mmol) in 125 mL of 80:20 methanol/acetic acid cooled to -40 °C with a dry ice-acetone bath. During the course of the addition, the temperature rose to -20 °C. After warming to r.t., most of the solvent was removed with a rotary evaporator. Water (300 mL) was added and the solution was made basic with NaOH and extracted with CH_2Cl_2 (2 x 90 mL). The combined extracts were washed with brine, dried over K_2CO_3, and evaporated. The residue was purified by chromatography on silica gel eluting with EtOAc/MeOH (1:1) to give racemic **Compound 8** as a pale yellow oil (9.9 g, 81%). ^{1}H NMR ($CDCl_3$) δ: 8.49 (d, J = 2.1 Hz, 1H), 8.45 (d, J = 1.8 Hz, 1H), 7.87 (t, J = 1.8 Hz, 1H), 4.14 (t, J = 7.6 Hz, 1H), 3.13 (ddd, J = 5.3, 7.6, 10.0 Hz, 1H), 3.02 (ddd, J = 6.8, 7.9, 10.0 Hz, 1H), 2.24-2.16 (m, 1H), 1.93-1.77 (m, 2H), 1.64-1.55 (m, 1H). ^{13}C NMR ($CDCl_3$) δ: 149.0, 146.6, 142.7, 136.6, 120.7, 59.1, 46.9, 34.5, 25.4. HRMS (MALDI-FTMS): calcd for $C_9H_{12}BrN_2$ (MH^+) 227.0184, found 227.0175.

EXAMPLE 5: Resolution of racemic 5-bromonornicotine Compound 8 via the α-methoxy-α-(trifluoromethyl) phenylacetate (MTPA) salts. A solution of (-)-MTPA (3.9 g, 16.7 mmol) in EtOAc (13 mL) was added to a solution of **Compound 8** (7.57 g, 33.3 mmol) in EtOAc (52 mL) with stirring. The mixture was allowed to stand at r.t. for 15 min, after

which time the crystalline product was collected by filtration to give (*R*)-isomer enriched crystals. Three recrystallizations from boiling acetonitrile yielded 4.1 g (54%) of colorless needles [(*R*)-5-bromonornicotine (-)-MTPA salt]. The filtrate was extracted with 1 N sulfuric acid (2 times). The acid extracts were combined, washed with ether, made basic with NaOH, and extracted with CH_2Cl_2. The organic layer was evaporated and purified on a silica gel column to give an (*S*)-isomer enriched oil. The oil was dissolved in EtOAc (25 mL) and treated with a solution of (+)-MTPA (3.9 g, 16.7 mmol) in EtOAc (13 mL) with stirring. After the solution was left standing for 15 min, the crystallized product was collected by filtration. Three recrystallizations from boiling acetonitrile yielded 4.0 g (52%) of colorless needles [(*S*)-5-bromonornicotine (+)-MTPA salt].

EXAMPLE 6: (*S*)-nornicotine Compound 2b. A suspension of (*S*)-5-bromonornicotine (+)-MTPA salt (2.39 g, 5.17 mmol) in ether (120 mL) was vigorously shaken with 1 M KOH (50 mL) in a separatory funnel. The ether layer was washed with 1 M KOH (50 mL), dried over anhydrous K_2CO_3 and evaporated. The residual oil was dissolved in EtOH (50 mL) containing Et_3N (1.25 mL) and hydrogenated at 1 atm with 10% Pd/C (500 mg). After 1 hr, the mixture was filtered through celite and the filter cake washed with EtOH. The filtrate was poured into 1 M K_2CO_3 (125 mL) which was then extracted with two 125 mL portions of CH_2Cl_2. After washing with brine (50 mL), the combined extracts were dried over anhydrous K_2CO_3 and evaporated. The residue was purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (7:1) to give **Compound (*S*)-2b** as a pale yellow oil (621 mg, 81% yield). ^{1}H NMR ($CDCl_3$) δ: 8.54 (d, J = 2.1 Hz, 1H), 8.42 (dd, J = 1.8, 5.0 Hz, 1H), 7.67 (dt, J = 1.8, 7.9 Hz, 1H), 7.19 (ddd, J = 0.6, 4.7, 7.9 Hz, 1H), 4.11 (t, J = 7.6 Hz, 1H), 3.15 (ddd, J = 5.6, 7.6, 10.0 Hz, 1H), 3.03-2.97 (m, 1H), 2.21-2.14 (m, 1H), 1.95-1.77 (m, 2H), 1.67-1.57 (m, 1H). ^{13}C NMR ($CDCl_3$) δ: 148.5, 148.1, 140.0, 134.0, 123.2, 59.9, 46.8, 34.2, 25.4. HRMS (MALDI-FTMS): calcd for $C_9H_{13}N_2$ (MH^+) 149.1079, found 149.1074. $[\alpha]^{23}_D$ = -35.8° (c = 2.184, MeOH). The (*R*)-**2c** isomer was prepared in a similar fashion from (*R*)-5-bromonornicotine (-)-MTPA salt. $[\alpha]^{23}_D$ = + 37.5° (c = 3.806, MeOH).

^{1}H NMR of a diastereomeric derivative was used to determine the enantiomeric excess of each isomer. A small amount of (*S*)-, (*R*)-, or *rac*-nornicotine (~10 mg) in CH_2Cl_2

(50 μL) was added Et_3N (30 μL, 0.215 mmol) and a solution of (*S*)- α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (800 μL, 0.1 M in CH_2Cl_2). The reaction mixture was mixed briefly and then evaporated. The residue was partitioned between EtOAc and water and the EtOAc layer was washed with brine, dried over Na_2SO_4 and evaporated. The starting material and by-products in the residue were removed by chromatography on silica gel, eluting with CH_2Cl_2/MeOH (50:1) to give the amide derivative as a colorless oil (TLC ≥90% pure). The 2'-H proton shifted from 4.11 ppm to 5.21 ppm for the (*S*)-nornicotine derivative and to 5.17 ppm for the (*R*)-nornicotine derivative. As indicated by the racemic sample, the patterns were different and clearly distinguishable. Neither diastereomer showed the presence of the other. To obtain a threshold of detection, each isomer was mixed with 1 mol% of the other isomer. Both samples showed a trace signal above noise at the expected location. Hence, it was possible to assign a lower limit of 98% ee to both (*S*)-nornicotine and (*R*)-nornicotine. (*S*)-nornicotine derivative (2'-H proton) δ: 5.21 (dd, J = 3.8, 6.2 Hz, 1H). HRMS (MALDI-FTMS): calcd for $C_{19}H_{20}F_3N_2O_2$ (MH^+) 365.1471, found 365.1474. (*R*)-nornicotine derivative (2'-H proton) δ: 5.17 (dd, J = 6.8, 7.9 Hz, 1H). HRMS (MALDI-FTMS): calcd for $C_{19}H_{20}F_3N_2O_2$ (MH^+) 365.1471, found 365.1483.

EXAMPLE 7: ***N*-[1-oxo-6-[(2*S*)-2-(3-pyridinyl)-1-pyrrolidinyl]hexyl]-β-alanine phenylmethyl ester Compound 9.** A solution of **Compound 14** (136 mg, 0.338 mmol) in acetonitrile (500 μL) was added to a mixture of **Compound 2b** (52.1 mg, 0.338 mmol) and diisopropylethylamine (DIEA) (117 μL, 0.676 mmol) in acetonitrile (850 μL) with stirring at r.t. After 18 hr, the mixture was evaporated and the residue purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (19:1) to give product **Compound 9** as a pale yellow oil (72 mg, 48% yield). ^1H NMR ($CDCl_3$) δ: 8.53 (br s, 1H), 8.46 (br d, 1H), 7.67 (br d, 1H), 7.36-7.31 (m, 5H), 7.23 (dd, J = 4.7, 7.9 Hz, 1H), 6.07 (br s, 1H), 5.12 (s, 2H), 3.50 (q, J = 5.9, 2H), 3.31 (dt, J = 2.1, 8.2 Hz, 1H), 3.23 (t, J = 8.2 Hz, 1H), 2.57 (t, J = 5.9 Hz, 2H), 2.43 (dt, J = 8.2, 11.7 Hz, 1H), 2.20-2.13 (m, 2H), 2.05 (t, J = 8.2 Hz, 2H), 2.07-2.01 (m, 1H), 1.97-1.85 (m, 1H), 1.85-1.76 (m, 1H), 1.69-1.60 (m, 1H), 1.53-1.45 (m, 2H), 1.43-1.36 (m, 2H), 1.32-1.13 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 172.9, 172.5, 149.4, 148.4, 135.6, 134.9, 128.6, 128.4, 128.2, 123.5, 67.6, 66.5, 54.0, 53.5, 36.6, 35.1, 34.7, 34.1, 28.4, 26.9, 25.4,

22.6. HRMS (MALDI-FTMS): calcd for $C_{25}H_{34}N_3O_3$ (MH^+) 424.2595, found 424.2607. $[\alpha]^{23}_D$ = -65.3° (c = 1.432, MeOH).

EXAMPLE 8: ***N*-[1-oxo-6-[(2*S*)-2-(3-pyridinyl)-1-pyrrolidinyl]hexyl]-β-alanine Compound 10 (NIC).** The benzylester **Compound 9** (51.4 mg, 0.122 mmol) in MeOH (2 mL) was hydrogenated with 10% Pd/C (9.3 mg) at 40 psi. After 90 min, the mixture was filtered through celite and the filter cake washed with MeOH. The filtrate was evaporated and the residue purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (3:2) to give hapten **Compound 10** as a pale yellow oil (26 mg, 66% yield). ^{1}H NMR (CD_3OD) δ: 8.53 (s, 1H), 8.45 (d, *J* = 3.5 Hz 1H), 7.93-7.90 (m, 1H), 7.44 (dd, *J* = 5.0, 7.9 Hz, 1H), 3.55 (t, *J* = 8.8 Hz, 1H), 3.49-3.43 (m, 1H), 3.38 (t, *J* = 6.8 Hz, 2H), 2.58-2.51 (m, 1H), 2.50-2.43 (m, 1H), 2.37 (t, *J* = 6.8 Hz, 2H), 2.32-2.24 (m, 2H), 2.11 (t, *J* = 7.3 Hz, 2H), 2.07-1.90 (m, 2H), 1.86-1.76 (m, 1H), 1.55-1.45 (m, 4H), 1.34-1.17 (m, 2H). ^{13}C NMR (CD_3OD) δ: 179.1, 176.0, 150.2, 149.7, 139.3, 138.0, 125.7, 69.5, 55.4, 54.8, 37.6, 37.6, 37.1, 35.2, 28.8, 28.0, 26.8, 23.5. HRMS (MALDI-FTMS): calcd for $C_{18}H_{28}N_3O_3$ (MH^+) 334.2131, found 334.2139. $[\alpha]^{23}_D$ = -71.0° (c = 0.534, MeOH).

EXAMPLE 9: ***N*-(1-oxo-6-bromohexyl)-β-alanine Compound 13.** A mixture of 6-bromohexanoic acid **Compound 11** (2.20 g, 11.4 mmol) and HBTU (International Peptides) (12.5 mmol) in DMF (15 mL) was stirred at 0 °C for 10 min. To the reaction mixture was added a solution of β-alanine benzyl ester *p*-TsOH salt **Compound 12** (Sigma) (4.00 g, 11.4 mmol) and N-methyl-morpholine (3.75 mL) in DMF (10 mL) at 0 °C with stirring. After 24 hr at r.t., the reaction mixture was poured into water and extracted with EtOAc. The EtOAc layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by chromatography on silica gel eluting with hexane/EtOAc (1:1) to **Compound 13** as a pale yellow oil (3.4 g, 84% yield). ^{1}H NMR ($CDCl_3$) δ: 7.37-7.34 (m, 5H), 6.02 (br s, 1H), 5.14 (s, 2H), 3.52 (q, *J* = 5.9 Hz, 2H), 3.39 (t, *J* = 6.8 Hz, 2H), 2.59 (t, *J* = 5.9 Hz, 2H), 2.13 (t, *J* = 7.6 Hz, 2H), 1.89-1.82 (m, 2H), 1.67-1.58 (m, 2H), 1.47-1.40 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 172.6, 172.4, 135.5, 128.5, 128.3, 128.2, 66.5, 36.2, 34.7, 34.0, 33.6, 32.3, 27.6, 24.6. HRMS (MALDI-FTMS): calcd for $C_{16}H_{22}BrNO_3Na$ (MNa^+) 378.0681, found 378.0693.

EXAMPLE 10: ***N*-(1-oxo-6-iodohexyl)-β-alanine Compound 14.** A mixture of **Compound 13** (1.00 g, 2.81 mmol) and sodium iodide (2.10 g, 14.1 mmol) in acetonitrile (6 mL) was vigorously stirred at r.t. for 18 hr. The progress of the reaction was followed by ^{1}H NMR. After completion, the mixture was evaporated and the residue partitioned between EtOAc and water. The EtOAc layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by chromatography on silica gel eluting with hexane/EtOAc (1:1) to give **Compound 14** as a colorless oil (1.11 g, 98% yield). ^{1}H NMR ($CDCl_3$) δ: 7.38-7.32 (m, 5H), 6.08 (br s, 1H), 5.13 (s, 2H), 3.51 (q, J = 6.2 Hz, 2H), 3.16 (t, J = 7.0 Hz, 2H), 2.58 (t, J = 6.2 Hz, 2H), 2.12 (t, J = 7.6 Hz, 2H), 1.84-1.76 (m, 2H), 1.64-1.56 (m, 2H), 1.42-1.34 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 172.6, 172.5, 135.6, 128.6, 128.4, 128.2, 66.5, 36.2, 34.7, 34.0, 33.0, 29.9, 24.4, 6.7. HRMS (MALDI-FTMS): calcd for $C_{16}H_{22}INO_3Na$ (MNa^+) 426.0537, found 426.0553.

EXAMPLE 11: ***Rac*-2-(3-pyridinyl)-1-pyrrolidinebutanoic acid phenylmethyl ester Compound 16a.** A solution of **Compound 20** (103 mg, 0.338 mmol) in acetonitrile (500 µL) was added to a solution of *rac*-nornicotine (50.0 mg, 0.338 mmol) and DIEA (117 µL, 0.676 mmol) in CH_3CN (850 µL) with stirring at r.t. After 18 hr, the reaction mixture was evaporated and the residue purified by chromatography on silica gel, eluting with CH_2Cl_2/MeOH (19:1) to give **Compound 16a** as a pale yellow oil (65 mg, 59% yield). ^{1}H NMR (CD_3OD) δ: 8.46 (br s, 1H), 8.38 (d, J = 4.7 Hz, 1H), 7.82-7.79 (m, 1H), 7.35-7.29 (m, 6H), 5.06 (d, J = 12.3 Hz, 1H), 5.00 (d, J = 12.3 Hz, 1H), 3.36-3.28 (m, 2H), 2.44 (dt, J = 8.2, 12.0 Hz, 1H), 2.32 (dt, J = 2.1, 7.0 Hz, 2H), 2.24-2.11 (m, 3H), 1.96-1.81 (m, 2H), 1.80-1.69 (m, 2H), 1.67-1.57 (m, 1H). ^{13}C NMR (CD_3OD) δ: 174.9, 149.9, 149.0, 141.7, 137.8, 137.6, 129.7, 129.4, 129.4, 125.5, 68.9, 67.3, 54.7, 54.5, 36.2, 32.9, 25.0, 23.7. HRMS (MALDI-FTMS): calcd for $C_{20}H_{25}N_2O_2$ (MH^+) 325.1911, found 325.1900.

(2*S*)-2-(3-pyridinyl)-1-pyrrolidinebutanoic acid phenylmethyl ester Compound 16b. Linker **Compound 20** (198 mg, 0.650 mmol), (*S*)-nornicotine **Compound 2b** (96.3 mg, 0.650 mmol), DIEA (226 µL, 1.30 mmol). Pale yellow oil (124 mg, 58% yield). $[\alpha]^{23}_D$ = -88.7° (c = 1.076, MeOH).

(2*R*)-2-(3-pyridinyl)-1-pyrrolidinebutanoic acid phenylmethyl ester Compound 16c. Linker **Compound 20** (198 mg, 0.650 mmol), (*R*)-nornicotine **Compound 2c** (96.3 mg, 0.650 mmol), DIEA (226 μL, 1.30 mmol). Pale yellow oil (131 mg, 62% yield). $[\alpha]^{23}_D$ = +87.5° (c = 1.072, MeOH).

EXAMPLE 12: *Rac*-2-(3-pyridinyl)-1-pyrrolidinebutanoic acid Compound 17a (*rac*-NOC). The benzylester **Compound 16a** (58.3 mg, 0.180 mmol) in MeOH (1.5 mL) was hydrogenated with 10% Pd/C (15 mg) using a balloon technique. After 75 min, the reaction mixture was filtered through celite and the filter cake washed with MeOH. The filtrate was evaporated and the residue purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (3:2) to give **Compound 17a** as a colorless oil (26 mg, 62% yield). ^{1}H NMR (CD_3OD) δ: 8.60 (d, *J* = 1.8 Hz, 1H), 8.53 (dd, *J* = 1.8, 5.0 Hz, 1H), 7.98 (dt, *J* = 2.1, 7.9 Hz, 1H), 7.48 (ddd, *J* = 0.9, 5.0, 7.9 Hz, 1H), 3.94 (dd, *J* = 7.6, 9.4 Hz, 1H), 3.71-3.65 (m, 1H), 2.85-2.69 (m, 3H), 2.43-2.38 (m, 1H), 2.33-2.26 (m, 1H), 2.17-1.98 (m, 4H), 1.83-1.71 (m, 2H). ^{13}C NMR (CD_3OD) δ: 179.7, 150.7, 150.6, 138.0, 136.1, 125.9, 69.5, 55.3, 54.6, 35.8, 34.4, 24.0, 23.3. HRMS (MALDI-FTMS): calcd for $C_{13}H_{19}N_2O_2$ (MH^+) 235.1441, found 235.1440.

(2*S*)-2-(3-pyridinyl)-1-pyrrolidinebutanoic acid Compound 17b [(*S*)-NOC]. The benzylester **Compound 16b** (120 mg, 0.370 mmol), 10% Pd/C (30 mg), reaction time 20 min. Colorless oil (63 mg, 73% yield). $[\alpha]^{23}_D$ = -15.7° (c = 1.202, MeOH).

(2*R*)-2-(3-pyridinyl)-1-pyrrolidinebutanoic acid Compound 17c [(*R*)-NOC]. The benzylester **Compound 16c** (95.0 mg, 0.293 mmol), 10% Pd/C (25 mg), reaction time 20 min. Colorless oil (41 mg, 59% yield). $[\alpha]^{23}_D$ = +13° (c = 0.822, MeOH).

EXAMPLE 13: Benzyl 4-bromobutanoate Compound 19. A solution of 4-bromobutanoic acid **Compound 18** (25 g, 0.15 mol), benzyl alcohol (21 g, 0.194 mol), and *p*-TsOH hydrate (1.3 g, 6.87 mmol) in cyclohexane (225 ml) was heated to reflux. The water was azeotropically removed with the aid of a Dean-Stark trap. After one hr, the stoichiometric amount of water was collected and the solution refluxed an additional hr.

After cooling to r.t., the solution was washed with sat. sodium bicarbonate, brine, dried over Na_2SO_4 and evaporated. The residue was distilled (bp 120-125 °C, 2 mmHg) to give **Compound 19** as a clear, colorless liquid (30 g, 78% yield). ^{1}H NMR ($CDCl_3$) δ: 7.40-7.32 (m, 5H), 5.13 (s, 2H), 3.46 (t, J = 6.5 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 2.23-2.16 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 172.3, 135.7, 128.5, 128.3, 128.2, 66.4, 32.7, 32.4, 27.7.

EXAMPLE 14: Benzyl 4-iodobutanoate Compound 20. A mixture of **Compound 19** (500 mg, 1.95 mmol) and sodium iodide (1.46 g, 9.75 mmol) in acetonitrile (4 mL) was vigorously stirred at r.t. for 18 hr. The progress of the reaction was followed by ^{1}H NMR. After completion, the reaction mixture was evaporated and the residue partitioned between EtOAc and water. The EtOAc layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by chromatography on silica gel eluting with hexane/CH_2Cl_2 (2:1) to give **Compound 20** as a colorless oil (466 mg, 79% yield). ^{1}H NMR ($CDCl_3$) δ: 7.39-7.34 (m, 5H), 5.14 (s, 2H), 3.23 (t, J = 6.8 Hz, 2H), 2.51 (t, J = 7.1 Hz, 2H), 2.19-2.12 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 172.0, 135.7, 128.5, 128.2, 128.1, 66.3, 34.7, 28.3, 5.4. MS (GCMS) $C_{11}H_{13}IO_2$ (Fw 304), *m/z* 304 (M^+).

EXAMPLE 15: [6-[(2*S*)-2-(3-pyridinyl)-1-pyrrolidinyl]hexyl]-carbamic acid 1,1-dimethylethyl ester Compound 21. A solution of **Compound 27** (425 mg, 1.30 mmol) in acetonitrile (2 mL) was added to a mixture of **Compound 2b** (200 mg, 1.35 mmol) and DIEA (470 μL, 2.70 mmol) in acetonitrile (4 mL) with stirring at r.t. After 15 hr, the reaction mixture was evaporated and the residue purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (30:1) to give **Compound 21** as a pale yellow oil (266 mg, 59% yield). ^{1}H NMR (CD_3OD) δ: 8.49 (s, 1H), 8.41 (d, J = 3.8 Hz, 1H), 7.85 (dt, J = 1.8, 7.9 Hz, 1H), 7.40 (dd, J = 5.0, 7.9 Hz, 1H), 3.36-3.29 (m, 2H), 2.96 (t, J = 7.0 Hz, 2H), 2.44 (dt, J = 8.5, 12.0 Hz, 1H), 2.29-2.07 (m, 2H), 2.13 (m, 1H), 1.96-1.85 (m, 2H), 1.72-1.63 (m, 1H), 1.46-1.35 (m, 13H), 1.30-1.18 (m, 4H). ^{13}C NMR (CD_3OD) δ: 158.7, 149.9, 149.0, 141.7, 137.7, 125.5, 79.9, 69.2, 55.7, 54.9, 41.4, 36.0, 31.1, 29.8, 29.0, 28.4, 27.9, 23.6. HRMS (MALDI-FTMS): calcd for $C_{20}H_{34}N_3O_2$ (MH^+) 348.2646, found 348.2630. $[\alpha]^{23}_D$ = -74.2° (c = 0.96, MeOH).

EXAMPLE 16: [6-[(5*S*)-2-oxo-5-(3-pyridinyl)-1-pyrrolidinyl]hexyl]-carbamic acid 1,1-dimethylethyl ester Compound 22. A solution of **Compound 21** (265 mg, 0.764 mmol) in dioxane (30 mL) was added to a mixture of $Hg(OAc)_2$ (1.22 g, 3.82 mmol) and aqueous EDTA (7.66 mL, 5 mmol/10 mL, pH 9.0) in water (39 mL) and the mixture refluxed with stirring for 2 hr. After cooling, the mixture was filtered through celite and the filter cake washed with a small amount of dioxane. The filtrate was extracted with CH_2Cl_2 and the organic layer washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (25:1) to give **Compound 22** as a pale yellow oil (95 mg, 34% yield). ^{1}H NMR (CD_3OD) δ: 8.52 (dd, J = 1.5, 5.0 Hz, 1H), 8.50 (d, J = 1.5 Hz, 1H), 7.78 (dt, J = 2.1, 7.9 Hz, 1H), 7.49 (ddd, J = 0.9, 5.0, 7.9 Hz, 1H), 6.54 (br s, 1H), 4.88-4.85 (m, 1H), 3.61-3.53 (m, 1H), 3.00-2.95 (m, 2H), 2.64-2.48 (m, 4H), 1.95-1.89 (m, 1H), 1.42-1.35 (m, 13H), 1.28-1.19 (m, 4H). ^{13}C NMR (CD_3OD) δ: 178.2, 158.7, 150.3, 149.4, 139.0, 136.9, 126.0, 79.9, 61.5, 42.1, 41.4, 31.3, 30.9, 29.3, 29.0, 27.9, 27.6, 27.5. HRMS (MALDI-FTMS): calcd for $C_{20}H_{31}N_3O_3Na$ (MNa^+) 384.2258, found 384.2273. $[\alpha]^{23}_D$ = -16.4° (c = 1.834, MeOH).

EXAMPLE 17: 4-oxo-4-[[6-[(5*S*)-2-oxo-5-(3-pyridinyl)-1-pyrrolidinyl]]hexyl]amino]-butanoic acid phenylmethyl ester Compound 23. A solution of **Compound 22** (91.7 mg, 0.254 mmol) in CH_2Cl_2 (500 µL) was treated with TFA (400 µL) at 0 °C. After completion, the mixture was evaporated with toluene (3 times). HBTU (111 mg, 0.292 mmol) was added to a solution of **Compound 29** (58.1 mg, 0.279 mmol) in DMF (500 µL) at 0 °C with stirring. After 10 min, a solution of the deprotected amine residue and N-methylmorpholine (167 µL, 1.52 mmol) in DMF (300 µL) were added at 0 °C with stirring. After 18 hr at r.t., the mixture was partitioned between EtOAc and water. The EtOAc layer was washed with brine, dried over Na_2SO_4, and evaporated. The residue was purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (35:1) to give **Compound 23** as a pale yellow oil (79 mg, 69% yield). ^{1}H NMR (CD_3OD) δ: 8.52 (dd, J = 1.5, 4.7 Hz, 1H), 8.49 (d, J = 1.5 Hz, 1H), 7.77 (dt, J = 1.8, 7.9 Hz, 1H), 7.48 (dd, J = 4.7, 7.9, Hz, 1H), 7.34-7.28 (m, 5H), 5.10 (s, 2H), 4.86-4.83 (m, 1H), 3.56 (dt, J = 7.6, 13.8 Hz, 1H), 3.09 (dt, J = 2.1, 7.0 Hz, 2H), 2.65 (t, J = 6.8 Hz, 2H), 2.63-2.51 (m, 4H), 2.47 (t, J = 6.8 Hz, 2H), 1.96-1.88 (m, 1H), 1.42-1.35 (m, 4H), 1.27-1.16 (m, 4H). ^{13}C NMR (CD_3OD) δ: 178.1, 174.2,

150.3, 149.4, 139.0, 137.8, 136.9, 129.7, 129.3, 129.3, 126.0, 67.5, 61.4, 42.0, 40.3, 31.6, 31.3, 30.7, 30.3, 29.2, 27.9, 27.5. HRMS (MALDI-FTMS): calcd for $C_{26}H_{33}N_3O_4Na$ (MNa^+) 474.2363, found 474.2384. $[\alpha]^{23}_D$ = -12.6° (c = 1.598, MeOH).

EXAMPLE 18: 4-oxo-4-[[6-[(5*S*)-2-oxo-5-(3-pyridinyl)-1-pyrrolidinyl]]hexyl]amino]-butanoic acid Compound 24 (COT). The benzyl ester **Compound 23** (77.0 mg, 0.171 mmol) in MeOH (2 mL) was hydrogenated with 10% Pd/C (15 mg) using a balloon technique. After 40 min, Pd/C was removed by filtration washing with MeOH. The filtrate was evaporated and the residue purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (3:1) to give **Compound 24** as a pale yellow oil (59 mg, 96% yield). ^{1}H NMR (CD_3OD) δ: 8.51 (br s, 2H), 7.79 (dt, *J* = 1.8, 7.9 Hz, 1H), 7.49 (dd, *J* = 5.0, 7.9 Hz, 1H), 4.88-4.85 (m, 1H), 3.57 (dt, *J* = 7.6, 14.1 Hz, 1H), 3.11 (dt, *J* = 1.2, 7.0 Hz, 2H), 2.63-2.4 (m, 8H), 1.97-1.89 (m, 1H), 1.46-1.35 (m, 4H), 1.30-1.17 (m, 4H). ^{13}C NMR (CD_3OD) δ: 179.7, 178.1, 175.5, 150.3, 149.4, 139.0, 136.9, 126.0, 61.4, 42.0, 40.4, 33.0, 31.3, 30.3, 29.2, 27.9, 27.5. HRMS (MALDI-FTMS): calc'd for $C_{19}H_{27}N_3O_4Na$ (MNa^+) 384.1894, found 384.1898. $[\alpha]^{23}_D$ = -14.2° (c = 1.186, MeOH).

EXAMPLE 19: (6-Hydroxyhexyl)-carbamic acid 1,1-dimethylethyl ester Compound 26. A mixture of 6-aminohexanol **Compound 25** (2.0 g 17.1 mmol) and di-t-butyldicarbonate (4.1 g, 18.8 mmol) in MeOH (40 mL) was stirred at r.t. for 90 min. The reaction mixture was evaporated and the residue purified by chromatography on silica gel eluting with CH_2Cl_2/MeOH (49:1) to give **Compound 26** as a pale yellow oil (3.7 g, 99%). ^{1}H NMR ($CDCl_3$) δ: 4.63 (br s, 1H), 3.58 (t, *J* = 6.5 Hz, 2H), 3.07 (q, *J* = 6.5 Hz, 2H), 2,12 (br s, 1H), 1.56-1.49 (m, 2H), 1.48-1.40 (m, 11H), 1.37-1.27 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 156.0, 79.0, 62.4, 40.3, 32.5, 29.9, 28.3, 26.3, 25.2. MS (ESI): $C_{11}H_{23}NO_3$ (Fw 217), *m/z* 240 (MNa^+).

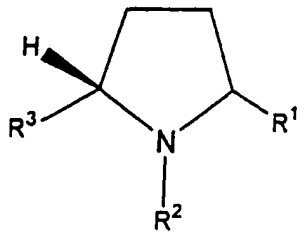
EXAMPLE 20: (6-Iodohexyl)-carbamic acid 1,1-dimethylethyl ester Compound 27. To a mixture of **Compound 26** (1.55 g, 7.13 mmol) and Et_3N (1.5 mL, 10.7 mmol) in CH_2Cl_2 (20 mL) in ice-cooled bath was added mesylchloride (607 μL, 7.8 mmol) with stirring. After 2 hr at r.t., the reaction mixture was washed with water and brine, dried over Na_2SO_4 and

evaporated. The residue was dissolved in acetonitrile (30 mL) and then sodium iodide (5.35 g, 35.7 mmol) was added at r.t. with stirring. After 18 hr, the mixture was evaporated and the residue partitioned between EtOAc and water. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated. The residue was purified by chromatography on silica gel eluting with hexane/EtOAc (10:1) and to give **Compound 27** as a colorless oil (1.8 g, 77% yield). ^{1}H NMR ($CDCl_3$) δ: 4.55 (br s, 1H), 3.16 (t, J = 6.8 Hz, 2H), 3.08 (q, J = 6.5 Hz, 2H) 1.80 (m, 2H), 1.48-1.35 (m, 13H), 1.34-1.28 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 155.9, 79.0, 40.4, 33.3, 30.1, 29.8, 28.3, 25.6, 6.9. MS (ESI): $C_{11}H_{22}INO_2$ (Fw 327), *m/z* 350 (MNa^+).

EXAMPLE 21: **Succinic acid monobenzyl ester Compound 29.** A mixture of succinic anhydride **Compound 28** (1.00 g, 10.0 mmol), benzyl alcohol (860 μL, 8.30 mmol) and DMAP (1.02 g, 8.30 mmol) in CH_2Cl_2 (30 mL) was stirred at r.t. for 18 hr. After this time, a solution of 5% Na_2CO_3 was poured into the reaction mixture and the layers separated. The aqueous layer was acidified with 1 M HCl and extracted with EtOAc. The EtOAc layer was washed with brine, dried over Na_2SO_4 and evaporated to give **Compound 29** as a colorless oil (1.63 g, 94%). ^{1}H NMR ($CDCl_3$) δ: 7.37-7.34 (m, 5H), 5.15 (s, 2H), 2.74-2.66 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 178.4, 172.0, 135.6, 128.5, 128.2, 128.1, 66.6, 28.9, 28.8. HRMS (MALDI-FTMS): calcd for $C_{11}H_{12}O_4Na$ (MNa^+) 231.0628, found 231.0627.

WHAT IS CLAIMED IS:

1. A nicotine immunogen of the formula



where R^1 is =O or H, R^2 is or $CO(CH_2)_nNH(CH_2)_2COOH$ $(CH_2)_nCONH(CH_2)_2COOH$, and R^3 is phenyl or pyridine and n is an integer from 1 to 10.

2. The immunogen of claim 1 wherein R^1 is H.

3. The immunogen of claim 1 wherein R^2 is $(CH_2)_nCONH(CH_2)_2COOH$.

4. The immunogen of claim 1 wherein R^3 is pyridine.

5. The immunogen of claim 1 wherein R^1 is H, R^2 is $(CH_2)_nCONH(CH_2)_2COOH$ and R^3 is pyridine.

6. The immunogen of claim 1 wherein n is from 3 to 7.

7. The immunogen of claim 1 wherein n is 5 or 6.

8. The immunogen of claim 1 operatively linked to an immunogenic carrier.

9. The immunogen of claim 8 wherein the carrier is keyhole limpet hemocyanin or bovine serum albumin.

10. A method of immunizing an animal against nicotine, the method comprising the step of administering to the animal an effective immunogenic amount of the immunogen of claim 1.

11. The method of claim 10 wherein R^1 is H, R^2 is $(CH_2)_nCONH(CH_2)_2COOH$ and R^3 is pyridine.

12. The method of claim 10 wherein the immunogen is operatively linked to an immunogenic carrier.

13. The method of claim 12 wherein the carrier is keyhole limpet hemocyanin or bovine serum albumin.

14. An antibody that immunoreacts with the immunogen of claim 1.

15. The antibody of claim 14 wherein R^1 is H, R^2 is $(CH_2)_nCONH(CH_2)_2COOH$ and R^3 is pyridine.

16. The antibody of claim 14 that also immunoreacts with nicotine.

17. The antibody of claim 14 that is a monoclonal antibody.

18. The monoclonal antibody of claim 17 designated as 3G3, 6C12, 13A3, 1B10, 5E8 or 9D9.

19. An anti-nicotine antibody made by a process that comprises the step of immunizing an animal with the immunogen of claim 1.

20. A method of immunizing an animal against nicotine comprising administering to the animal an effective immunogenic amount of the antibody of claim 14.

21. A pharmaceutical composition comprising the immunogen of claim 1 together with a physiological diluent.

22. A pharmaceutical composition comprising the antibody of claim 14 together with a physiological diluent.

Figure 1

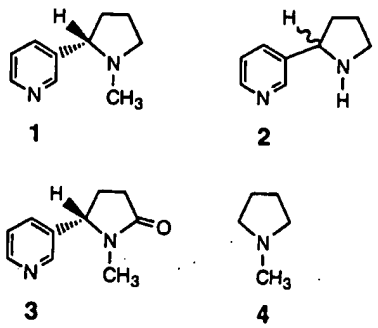
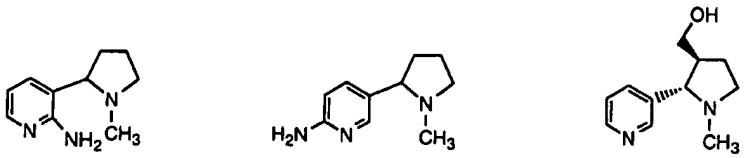


Figure 2



A variety of linkers attached at the pyridyl amino group of **A** and **B** have been reported. The hydroxy group of **C** has generally been succinylated in order to couple the final hapten to carrier proteins.

Figure 3

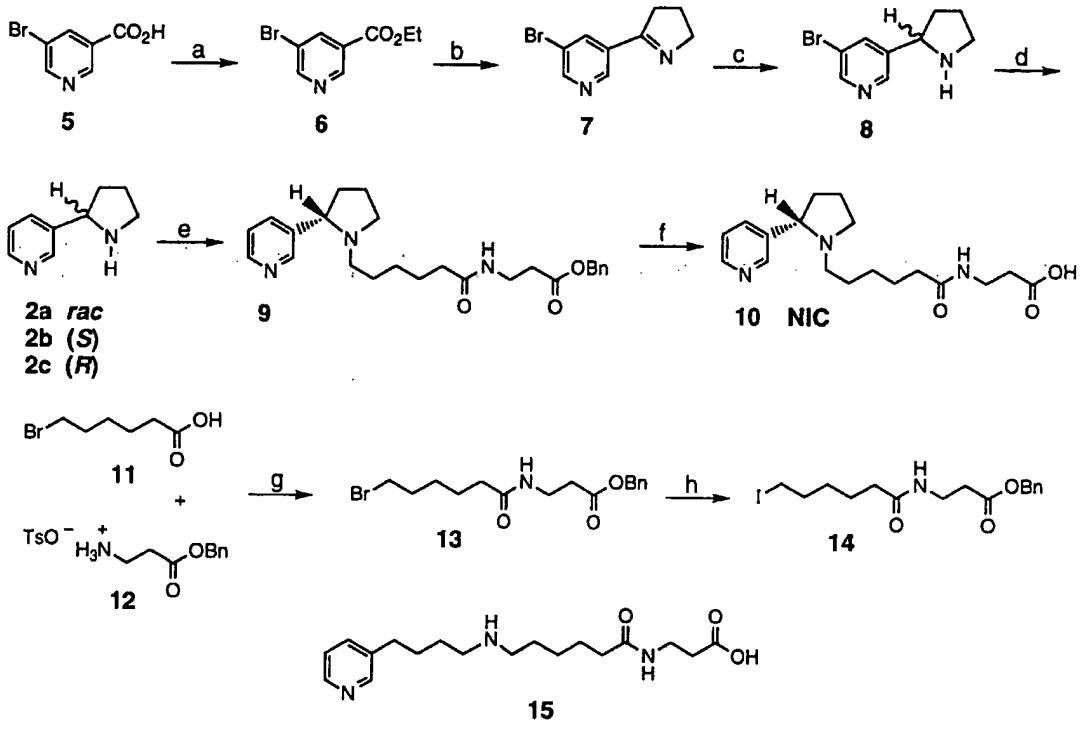

Br
CO2H
a
CO2Et
b
c
d
5
6
7
8
e
f
OBn
OH
2a rac
2b (S)
2c (R)
9
10 NIC
11
g
h
TsO⁻
H3N
12
13
14
15

Figure 4

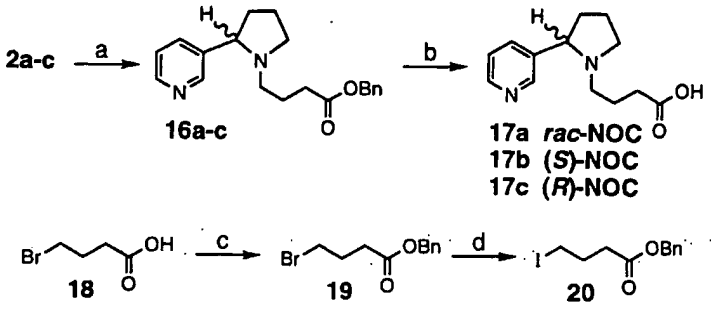


Figure 5

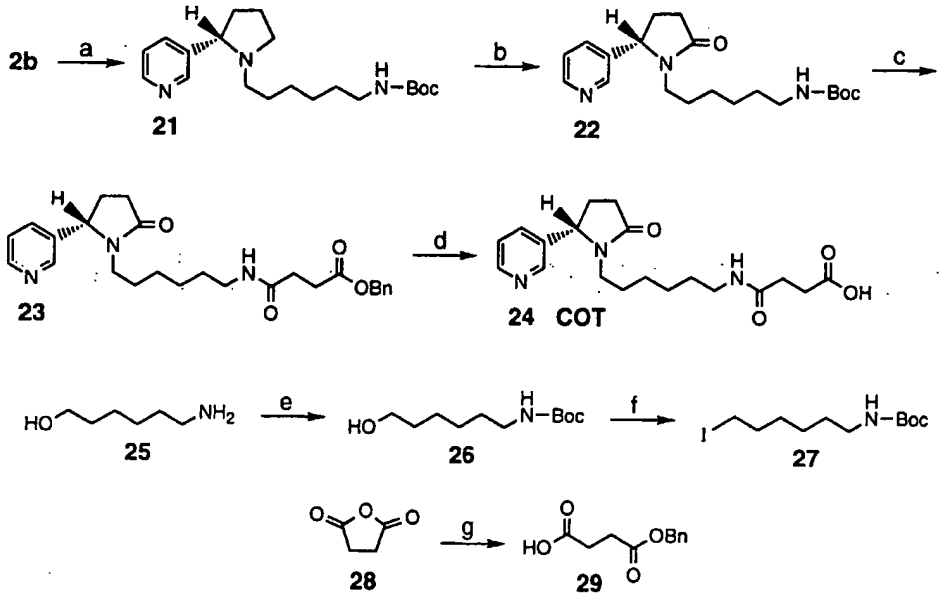


Figure 6

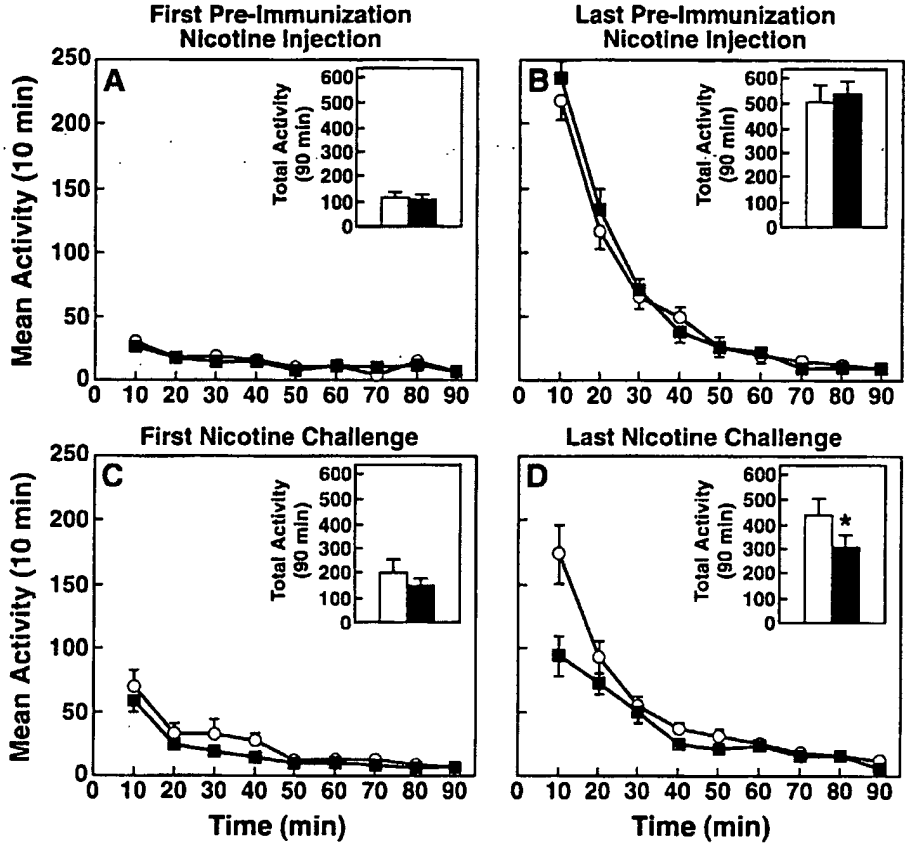
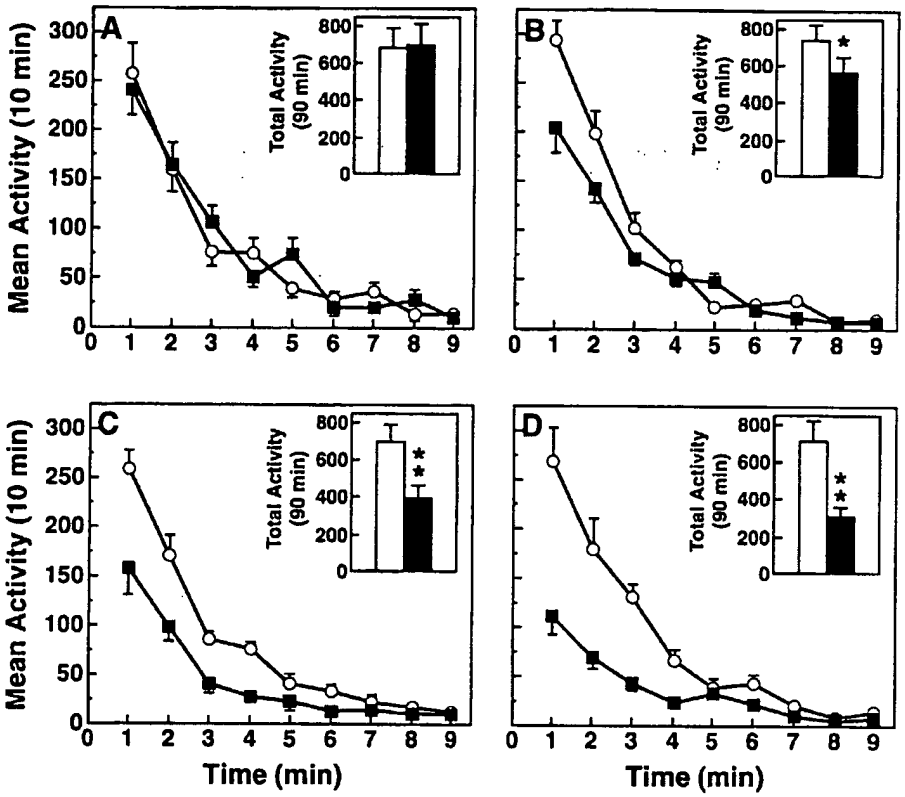

First Pre-Immunization
Nicotine Injection
Last Pre-Immunization
Nicotine Injection
First Nicotine Challenge
Last Nicotine Challenge
A
B
C
D
Mean Activity (10 min)
Time (min)
Total Activity
(90 min)
0 10 20 30 40 50 60 70 80 90
0 50 100 150 200 250
0 100 200 300 400 500 600
*

Figure 7


A
B
C
D
Mean Activity (10 min)
Time (min)
Total Activity (90 min)
0
50
100
150
200
250
300
0 1 2 3 4 5 6 7 8 9
0
200
400
600
800
*
**

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WO 02/058635 A3

(54) Title: NICOTINE IMMUNOGENS AND ANTIBODIES AND USES THEREOF

(57) Abstract: Immunogens useful in the production of anti-nicotine antibodies are provided. Anti-nicotine antibodies that immunoreact with the immunogens and nicotine and uses of the immunogens and antibodies in treating nicotine addiction are also provided.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/02146

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07D 401/04, 207/16; C07K 16/44; A61K 31/405, 31/44, 39/385, 39/395
US CL : 514/343, 424, 428; 424/141.1, 175.1; 538/540, 550; 546/278.4, 276.4, 279.1, 279.4; 530/388.9, 389.8, 403, 405

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/343, 424, 428; 424/141.1, 175.1; 538/540, 550; 546/278.4, 276.4, 279.1, 279.4; 530/388.9, 389.8, 403, 405

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, CAS ONLINE: structure searches; search terms: hapten, immun?, antibod?, antigen?, ELISA, KLH, BSA, OVA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	CASTRO. A. et al. Characterization of Antibodies to Nicotine. Biochemical Archives. 1985, Vol. 1, pages 173-183, FIGURE 1 and page 175, Immunization.	14-19, 22 --- 1-13, 20, 21
X --- Y	WO 00/32239 A1 (NABI) 08 June 2000 (08.06.00), claims 1-35.	14-19, 22 --- 1-13, 20, 21
X --- A	US 5,876,727 A (SWAIN et al) 02 March 1999 (02.03.1999), FIG. 17B; Fig. 18: PS-54; claims 1-18.	14-19, 22 --- 1-13, 20, 21
X --- A	WO 93/23076 A1 (THE JOHNS-HOPKINS UNIVERSITY) 25 November 1993 (25.11.93), claims 1 and 3.	14-19, 22 --- 1-13, 20, 21

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2002 (29.08.2002)	12 DEC 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Long V. Le Telephone No. (703) 308-1234

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PCT/US02/02146

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/61054 A1 (INDEPENDENT PHARMACEUTICA AB) 02 December 1999	14-19, 22
---	(02.12.99), claims 1-9.	---
A		1-13, 20, 21